Strategic drug analysis in fed-state gastric biorelevant media based on drug physicochemical properties

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Abstract: Milk-based media such as the Fed State Simulated Gastric Fluid (FeSSGF) are commonly used in order to simulate the *in vivo* properties of the fed state stomach. Due to the lack of a specific guideline for standardised sample clean-up in these media, the aim of the current study was to develop an optimum protocol for the extraction and quantification of drugs from the fed state gastric medium based on the APIs' physicochemical properties (lipophilicity, ionisation, aqueous solubility and protein binding). Two different extraction techniques, protein precipitation (PP) and solid phase extraction (SPE) were assessed. A pilot study in six model drugs was performed, with tests using seven different protein precipitation reagents at four different medium:reagent ratios and two drug concentrations as well as different solid phase extraction cartridges and elution protocols. % recovery was analysed using partial least squares (PLS) regression so as to determine the physicochemical parameters affecting the drug percentage recovered. For protein precipitation protocols, drug concentration, selection of protein precipitation reagent and ratio added to the medium significantly affected drug % recovery from FeSSGF ($p < 0.05$). The same applied for the selection of elution solvent and cartridge type for solid phase extraction. Optimum protocols using MeOH, ACN and 10% w/v TCA at a 1:2 FeSSGF:reagent ratio were effective to a larger group of drugs of a wide range of lipophilicity and ionisation, with ACN being the most effective in the whole range of log P values (-0.56-8.81). Solid phase extraction was proven to be effective for compounds of poor to moderate lipophilicity (log $P < 4$), with extremely hydrophobic compounds demonstrating lower % recovery values (down to 10% recovery). PLS demonstrated that only for 10% w/v TCA (protein precipitation) and HLB (solid phase extraction) can the effect of key drug physicochemical properties on the final amount of drug recovered be accurately predicted.

Keywords: Fed state; Protein precipitation; Solid phase extraction; Biorelevant media; Drug analysis; Physicochemical properties

Abbreviations

ACN:acetonitrile, **API**:active pharmaceutical ingredient, **BA/BE**:Bioavailability/bioequivalence, CV:coefficient of variation, **EtOH**:ethanol, **FeSSGF**:Fed state simulated gastric fluid, **GF/D**:glass microfiber, **HLB**:hydrophilic-lipophilic balance, **log aq sol**:logarithm of drug aqueous solubility in mg/mL, **LOQ**:limit of quantification, **MeOH**:methanol, **MLR:**multiple linear regression, **PLS:**partial least squares, **PP**:protein precipitation, **prot b fr:**drug protein bound fraction in serum proteins, **RC**:regenerated cellulose, **SPE**:solid phase extraction, **PLS:**partial least squares, **TCA**:trichloroacetic acid, **TFA**:trifluoroacetic acid, **union fr**:drug unionised fraction at pH = 5

Drug abbreviations: **ATE**:atenolol, **ATORV**:atorvastatin calcium, **ATOV**:atovaquone, **AZITH**:azithromycin, **CEL**:celecoxib, **DAN**:danazol, **FUR**:furosemide, **ITR**:itraconazole, **KET**:ketoconazole, **LAP**:lapatinib, **METF**:metformin hydrochloride, **METOP**:metoprolol tartrate, **NIF**:nifedipine, **PAR**:paracetamol, **PRAV**:pravastatin sodium, **PROP**:propafenone hydrochloride

1. Introduction

The presence of food in the gastric environment as a factor affecting drug dissolution and absorption has been extensively discussed in the literature over the last 30 years. Even though most drugs are mainly absorbed in the small intestinal environment, the role of the gastric environment is equally important; the stomach acts as a reservoir, with the presence of food having a significant influence on the absorption of drugs through various mechanisms such as delayed gastric emptying [1], increased gastric residence time [2] and interaction of drugs with meal components [3].

The FDA proposes the use of high fat standard meals for the determination of a drug's food effect, as meals of high caloric and fat content can stimulate bigger changes in the gastrointestinal physiology and consequently have a more pronounced effect on drug bioavailability when this is affected by the presence/absence of food [4]. In theory, the optimal medium for the determination of food effect *in vitro* would be a homogenised standard meal, similar to the ones which have been successfully used for *in vivo* studies [5]. Due to difficulties in aspiration and handling of such a medium though [6], a range of biorelevant dissolution media has been developed in order to simulate the *in vivo* conditions of the fed state stomach. These media were developed with an aim of having the same physicochemical properties with the standard meal recommended by FDA for BA/BE studies [4].

Milk and milk-based media have been used as dissolution media for gastric fed state simulation for more than twenty years. Despite milk's simplicity and convenience though, its energy content differs to that of a standard high-fat breakfast used in BA/BE studies [7] and does not accurately simulate the gastric fed state conditions, because of deviations in osmolality and buffer capacity compared to the FDA-proposed standard meal [5]. Moreover, its pH value is significantly higher (pH ≈ 6.5) than the gastric pH after a meal administration (5.8 ± 0.2 at

50% of the meal emptied by the gastric compartment, liquid meal administered) [8]. In order to improve the gastric environment simulation, a milk-based medium called Fed State Simulated Gastric Fluid (FeSSGF) was developed. This medium consists of 3.5% fat milk, diluted with acetate buffer at 1:1 ratio [6, 9]. Three FeSSGF versions with different pH values have been used so as to mimic the three phases of gastric digestion with the pH values being 6.4, 5.0 and 3.0 for the early, middle and late phases respectively [10]. Despite its limited ability to simulate the gastric fed conditions at each point of ingestion, middle phase FeSSGF is used as a compromise for the reflection of the sum of gastric physiological events during ingestion [10]. Unlike aqueous media, laborious techniques are usually required for extraction of drug from these milk-based media. A study where the extraction process was avoided [11] used an ion selective electrode (ISE) sensor system with two electrodes constantly in the dissolution vessel offering the possibility of a continuous dissolution profile. The method though was limited to the analysis of ionised drugs, it required a complicated correction of the baseline and was unable to analyse compounds of extremely low aqueous solubility.

Protein precipitation is the most frequently used sample clean-up technique, in which an organic reagent is added to the milk-based medium, followed by a centrifugation and a filtration step. Organic reagents like acetonitrile (added at 1:1 [12] and 1:2 [13, 14] medium:reagent ratios), isopropanol (1:1 ratio [15]) and ethyl acetate (1:4 ratio [16]) have been successfully used so far, but the rationale regarding the selection of the optimum reagent has not been yet clarified.

Solid phase extraction (SPE) is another extraction technique widely used for the extraction of drugs from heterogeneous media and biological fluids such as whole blood [17], plasma [18], urine [19], and milk [20], often preceded by a protein precipitation step. SPE cartridges consist of a polypropylene tube with the sorbent placed between two porous frits. Most cartridges are either comprised of bonded silica phases, similar to the material of the

reversed phase HPLC columns but with bigger diameter particles (10-60 μm), or of polymeric resins (e.g. polystyrene-divinylbenzene) [21].

So far, there has been no specific guideline for the treatment of each compound according to its characteristics and every drug is examined separately as far as its effective extraction and quantification in fed gastric biorelevant media are concerned [22]. Due to the absence of a general *in vitro* predictive test, the aim of this study was the development of an optimised protocol for drug quantification in fed gastric biorelevant media, towards the build-up of an *in vitro* predictive test of food effect observed *in vivo*. To achieve the above, an analytical protocol in milk-based fed gastric biorelevant media dictating the optimum sample treatment maximising the method sensitivity was developed, providing an analytical roadmap guide according to the drug's physicochemical properties.

For the current study, a series of compounds of a wide range of lipophilicity and ionisation were selected as model compounds for the development of the analytical protocol, assessing the efficiency of the two extraction techniques mentioned above; protein precipitation and solid phase extraction. An extension of the multiple linear regression model (MLR), partial least squares (PLS) regression was used to understand the impact of certain variables (drug lipophilicity, aqueous solubility, drug ionisation properties and protein binding) on the performance of two commonly used sample clean-up techniques for drugs dissolved in milk-based fed state biorelevant media. Its main advantage compared to the latter is its ability to analyse data with collinear independent variables [23]. To our knowledge, this is the first time the creation of a general analytical guideline for a range of compounds in fed gastric media is being attempted. Moreover, an innovation of the study is the use of partial least squares regression in order to define the critical parameters which affect the efficacy of protein precipitation and solid phase extraction in fed gastric media, justifying their selection with statistical tools.

2. Materials and Methods

2.1. Materials

Furosemide (≥ 98% (HPLC), (±)-metoprolol (+)-tartrate salt (≥ 98% (titration)), 1,1-dimethylbiguanide hydrochloride (metformin hydrochloride, 97%), danazol (≥ 98%), itraconazole (≥ 98% (TLC)), propafenone hydrochloride (≥ 98% (HPLC)), celecoxib (≥ 98% (HPLC)), azithromycin (≥ 95% (NT)) and atovaquone (≥ 98% (HPLC)) were all purchased from Sigma-Aldrich, UK. Nifedipine (98 to 102% (on dried substance)), paracetamol (97.5% min. (HPLC)), atorvastatin calcium (pharmaceutical secondary standard; traceable to USP, PhEur), atenolol (≥ 98% (TLC)) and ketoconazole (inclusive between 98%) were all purchased from Fisher Scientific, UK. Pravastatin sodium (≥ 98%) and lapatinib (≥ 99% (HPLC)) were purchased from Carbosynth, UK. MK-C1, MK-C2, MK-C3 and MK-C4 were provided by Merck & Co, INC, US.

Sodium chloride, sodium hydroxide, sodium dodecyl sulphate, sodium acetate trihydrate, dipotassium hydrogen orthophosphate, potassium dihydrogen phosphate, ammonium acetate, hydrochloric acid 37% glacial acetic acid ≥ 99% and trichloroacetic acid 10% w/v were all purchased from Fisher Scientific, UK. HPLC grade methanol, ethanol, acetonitrile, acetone, trifluoroacetic acid (≥ 99%) were all purchased from Sigma- Aldrich, UK.

3.6% fat UHT milk was commercially purchased (Sainsbury's, UK).

Cronus 13 mm regenerated cellulose (RC) syringe filters 0.45 µm were purchased from LabHut Ltd, UK, Whatman 13 mm glass microfiber syringe filters 2.7 µm (GF/D) from Fisher Scientific, UK and SPE cartridges (Sep-Pak tC_{18} 3 cc Vac Cartridge, 500 mg Sorbent per Cartridge, 37-55 µm Particle Size, Sep-Pak C_8 3 cc Vac Cartridge, 500 mg Sorbent per

Cartridge, 37-55 µm Particle Size and Oasis HLB 1 cc Vac Cartridge, 30 mg Sorbent per Cartridge, 30 µm Particle Size) from Waters, UK.

2.2. Instrumentation

All samples were analysed in an HPLC system consisting of an Agilent 1200 series binary pump (G1312A), an Agilent 1200 series DAD detector (G1315D), an Agilent 1200 series autosampler (G1329A), an Agilent 1200 series controller (G1316A) and a Chemstation software (Agilent Technologies, Santa Clara, United States).

A pH meter Mettler-Toledo AG (model SevenCompact pH/Ion S220, Schwerzenbach, Switzerland), a centrifuge Hereus Biofuge Primo R (Thermo Scientific, Hanau, Germany), a vortex mixer Rotamixer (HTZ, Chesire, UK) and, a UV-Vis Thermo Spectronic Helios Gamma spectrophotometer (Thermo Scientic, UK) were used.

2.3. Fed state medium selection

Fed State Simulated Gastric fluid (FeSSGF) was selected as the working medium due to its simplicity in its preparation and stability for 72 h [10]. Its buffer capacity, osmolality and surface tension values are in total closer to the values measured *in vivo* after the administration of a standard meal than the equivalent properties of milk, which has been extensively used as a gastric fed state medium in dissolution studies [24]. Finally, as it is less viscous than milk, its handing and loading/elution from the SPE cartridges was feasible without back pressure.

2.4. Medium preparation

Fed State Simulated Gastric Fluid (FeSSGF) was prepared according to Jantratid et al. [10], by mixing 3.6% fat milk and acetate buffer pH = 5 (17.12 mM CH_3COOH, 29.75 mM CH_3COONa, 237.02 mM NaCl in the medium) at a 1:1 volume ratio. For the preparation of 1 L of medium, 500 mL milk and 480 mL buffer were mixed under constant stirring using a

177 magnetic stirrer. pH was adjusted to 5 with 1 N HCl and the volume was adjusted to 1 L with
178 the buffer.

179 **2.5. Pilot study and selection of optimum conditions**

180 20 drugs of a wide range of physicochemical properties (lipophilicity, ionisation,
181 aqueous solubility and protein binding) were selected as model compounds (Table 1). 6
182 compounds were selected for the pilot study in order to assess the optimum extraction
183 techniques and protocols and determine the parameters affecting the extraction technique's
184 efficiency. The compounds selected for the pilot study were hydrophilic to extremely lipophilic
185 (log P = -0.56–6.20) and included acids, bases and a neutral compound. Each compound's %
186 absolute recovery was expressed as described in the equation (Eq. 1) below,

187 $$\%\ \text{absolute recovery} = \frac{\text{Area of peak of filtered aliquot}}{\text{Area of peak of standard solution of equivalent concentration in acetate buffer or MeOH: acetate buffer}} \quad \text{(Eq. 1)}$$

188 where filtered aliquot denotes the filtered drug solutions after protein precipitation or elution
189 from the SPE cartridge.

190 In protein precipitation, four organic and three aqueous reagents were tested to determine
191 the highest % absolute recovery values: methanol (MeOH), ethanol (EtOH), acetonitrile (ACN)
192 and acetone [organic reagents] and 2M HCl, 10% w/v trichloroacetic acid (TCA) and 10% w/v
193 trifluoroacetic acid (TFA) [aqueous reagents]. Four different FeSSGF:precipitation reagent
194 ratios (1:1, 1:2, 1:3 and 1:5) were tested to determine possible differences in % absolute
195 recovery and two different concentrations (a "high and a "low", defined below Table 1) in order
196 to assess the method efficiency at a range of drug concentrations. The parameter assessed in
197 SPE was elution volume, using tC_{18} cartridge (2 mL and 5 mL).

198 The efficiency of two extraction techniques [protein precipitation (PP) and solid phase
199 extraction (SPE)] was investigated in the pilot study with the optimised protocol being applied

to all compounds in Table 1. The optimum conditions (drug concentration, reagent and ratio) were selected and applied to all model compounds. The minimum efficiency limit for the pilot study was arbitrarily set to 50% (absolute recovery > 50%) [the 50% threshold was selected in order to obtain a final drug concentration in the extracted medium of not more than ~10 times lower than the initial drug concentration in the FeSSGF, and keep method sensitivity as high as possible]. Higher ratios (1:1, 1:2 were generally preferred due to higher method sensitivity (no need for dilution to overcome peak fronting). Similarly, for SPE, lower elution volume was preferred in case of similar recovery values. A 15% limit was set as the acceptable threshold for % CV in the final study with all the compounds tested under the optimum extraction conditions. The limit was selected in line with the regulatory requirements for acceptable variability in recovered samples as proposed by the EMA [51] and FDA [52] in the guidelines for bioanalytical method validation.

2.6. HPLC analysis

Stock solutions of the drugs were prepared in MeOH, EtOH, ACN or H_2O, based on the drug solubility in the above solvents. Calibration standards were prepared in organic solvent:"blank" acetate buffer 1:1 (pH adjusted to 5) mixture or acetate buffer pH 5, (where organic solvent is MeOH, EtOH or ACN, according to drug solubility in organic solvents). The drugs were analysed in HPLC with published HPLC methods (or modifications of published methods) which are stated in Table 2.

Adsorption studies were performed in triplicate for each model drug for all types of filters used. No adsorption issues were observed for the drugs studied.

2.7. FeSSGF solubility studies

Where FeSSGF or milk solubility data was not available in the literature, drug 24 h-solubility values in FeSSGF were determined by using a modification of a protocol using the shake-flask method [15]. The solubility of the model compounds was determined by weighing excess amounts of the drug into 5 mL Eppendorf tubes, followed by the addition of 5 mL of FeSSGF. The samples were left to equilibrate in a shaking water bath at 37 °C for 24 hours, and then filtered through a GF/D filter of 2.7 µm pore size. 1 mL of ACN was added to 0.5 mL of the filtered sample, vortexed for 30 sec and centrifuged (15 min, 8000 rpm, 4 °C). The supernatant, was filtered through a 0.45 µm RC filter, diluted and analysed using HPLC. Drug was quantified against calibration standards in FeSSGF which had undergone the same treatment as the sample. Each measurement was performed in triplicate.

2.8. Protein precipitation (PP)

2.8.1. Protein precipitation methodology

1 mL of working solution of each drug in FeSSGF was placed in a plastic centrifuge tube. A volume of the protein precipitation reagent according to the FeSSGF:protein precipitation reagent ratios as defined below (1, 2, 3, 5 mL) was added. The mixture was vortexed at full speed for 30 sec and centrifuged at 8000 rpm (9800 × g) for 15 minutes (4 °C). The supernatant was filtered through a 0.45 mm RC filter and assayed. The sample was diluted with acetate buffer or MeOH:acetate buffer 1:1 when diluent was more highly eluting than the mobile phase and peak shape needed to be improved.

2.8.2. Matrix interference

Full scans of the supernatants of the six drugs used in the pilot study plus atovaquone (a compound which demonstrated big differences in recovery between the three optimum reagents used.), with MeOH, acetonitrile and 10% w/v TCA used as protein precipitation reagents, were performed using the diode array detector of the HPLC instrument, to determine possible interferences from the medium and precipitation reagents in drug analysis. Scans were performed over a range from 190 to 400 nm. Standards were prepared in a mixture comprising one part of buffer and two parts of PP reagent so as to maintain the same amount of precipitation reagent as the extracted FeSSGF samples with the selected reagents. Spectra of a supernatant after proteins were precipitated with a specific reagent and spectra of the same drug, dissolved in 1 part of acetate buffer pH = 5 and 2 parts of the selected protein precipitation reagent were normalised to peak intensity and superimposed using the "best possible match of the entire spectrum" mode in Chemstation software. Chromatograms of drug samples were compared against "blank" samples (one part of FeSSGF + two parts of protein precipitation reagent) to determine possible interferences for the medium.

2.9. Solid phase extraction (SPE)

Three different types of cartridges were used: tC_{18} (500 mg bed weight), HLB (30 mg bed weight), C_8 [(500 mg bed weight)-used for the extraction of metformin hydrochloride only]. The extraction cartridges were conditioned by washing with 5 mL [tC_{18} (trifunctional octadecyl silica), C_8] or 1 mL MeOH (HLB), followed by 5 mL and 1 mL of H_2O respectively. 1 mL of FeSSGF was loaded and the columns were 5 washed with 5 mL and 1 mL of H_2O respectively. The drugs were eluted with 5 or 2 mL MeOH:H_2O 70:30 (tC_{18} and C_8 cartridges) or 1 mL MeOH (HLB cartridges).

As in the case of protein precipitation, a pilot study with the initial six compounds was performed and the optimal conditions of the parameters examined were applied for the rest of the model compounds. Modifications of the above protocols were performed in cases of % absolute recovery values < 50%, with different approaches according to each drug's physicochemical properties and are described in detail in *SPE protocol optimisation* part of *Results and Discussion* section. Protocols were optimised by: ***modifications in cartridge conditioning*** (a. use of an ion-pair reagent, b. conditioning of the cartridge with an acid or a base so as to improve its retention characteristics) or ***modifications in elution*** (a. use of different elution solvents, b. addition of acid or base in elution solvent so as to increase its elution strength). Specifically:

Metformin: A C_8 cartridge which retains hydrophilic compounds better was used and either the washing step was omitted or the HLB cartridge was pre-treated with 2 mM Sodium dodecyl sulphate (SDS) solution before the loading step. SDS was selected based on the hypothesis that due to the stationary phase's chemistry, the equilibration of the HLB cartridges with an ion pair reagent would lead to the retention of the drug to the cartridge through development of hydrophobic interactions between drug and cartridge with the complex easily be broken during the elution of the drug with an organic elution solvent [69]. *Atovaquone, Lapatinib, MK-C1, MK-C2, MK-C3, MK-C4:* Elution with MeOH for the more effective disruption lipophilic interactions between the drug and the lipophilic chains of the tC_{18} cartridge. *Itraconazole:* Pre- treatment of the cartridge (HLB) with 0.01 M NaOH (to retain the drug (weak base) on the cartridge and elution with 0.25 M formic acid in MeOH for a more efficient elution in its ionised form.

2.10. Statistical Analysis

Comparisons were performed in order to assess significant changes in drug recovery using different precipitation reagents, medium:reagent ratios, drug concentration, different SPE cartridges and elution volumes. For protein precipitation, % absolute recovery and correlation with added protein precipitation reagent, (FeSSGF:reagent ratio) and drug concentration were evaluated in the context of a multiple way Analysis of Variance (ANOVA) (Statgraphics v. XVI, StatPoint Technologies Inc, US) with a post-hoc Bonferroni test. In solid phase extraction, effect of different elution volumes and cartridges on drug % absolute recovery were compared using a two-tailed t-test. (Statgraphics v. XVI). Comparisons where $p < 0.05$ suggested a statistically significant difference.

The absolute % drug recovery using different protein precipitation or SPE protocols was correlated to drug physicochemical properties by partial least squares (PLS) regression using the XLSTAT software (Microsoft, US). The parameters evaluated were: lipophilicity (log P), log aqueous solubility in mg/mL, drug unionised fraction at pH = 5, acid/base properties and drug protein bound fraction in plasma proteins (drug bound fraction to plasma proteins was used as an indicator of protein affinity due to the lack of available data in milk proteins in the literature). The physicochemical properties selected as independent variables were decided on the basis of their potential effect on drug distribution in the aqueous and lipid phases of the medium and its interaction with milk proteins. PLS regression analysis was performed with % recovery of the three reagents used for the extraction of the 20 model compounds being the dependent variable. Selected interactions were also included in the model (log P*log aqueous solubility, log P*acid/base properties, aqueous solubility*drug unionised fraction, aqueous solubility*acid/base properties, unionised fraction*acid/base properties). The model quality was evaluated on the square of the coefficient of determination (R^2) and goodness of prediction (Q^2). R^2 and Q^2 values close to 1 refer to a model of good fit and

prediction power respectively while a difference lower than 0.2-0.3 between them is indicative of a successful model [70]. Full cross-validation (leave-one-out procedure) was used to develop and evaluate the regression model. The optimum number of calibration factors for each model was selected based on the model's optimum predictability (Q^2) and predicted residual error sum of squares (PRESS). A Q^2 value > 0.5 is generally considered acceptable for good model predictability [71]. Lower PRESS values indicate better prediction [72] with the number of latent variables where PRESS starts increasing indicating the number of variables which to be retained in the model [73]. The standardised coefficients of the factors plotted indicate the relative positive/negative effect of their corresponding variables on the % drug recovery (response value). High standardised coefficients for variance X have a big positive or negative effect on response Y. The importance of each parameter was evaluated by its variable importance in projection (VIP) value. Values above 1.0 are considered to have a significant effect on the dependent variable, whereas values < 0.7-0.8 are not of significance for the prediction of the dependent variable [70].

2.11. Roadmap design

The roadmaps leading to selection of optimal protein PP and SPE protocols for drug analysis were constructed combining the results from the complete study for the 20 model drugs (and selected PP and SPE conditions) and the variables affecting the drug percentage recovered, as demonstrated by the PLS regression analysis. Only models with Q^2 values > 0.5 were considered for the roadmap design. For protein precipitation, optimum conditions were selected on the basis of absolute % recovery. If absolute % recovery was > 85% for more than one reagent, MeOH or 10% w/v TCA were preferred over ACN, as they give peaks of better shape without the need of dilution. The 85% threshold was only used as a selection criterion, between protein precipitation obtained with acetonitrile or methanol. The limit was based on the method

capability as both methods gave recoveries consistently close to 100% for most drugs. Therefore, the 85% (100% - acceptable 15% CV, in line with the regulatory guidelines) [51, 52] was set in order to select the optimum protocol according to its maximum potential.

3. Results and Discussion

3.1. Drug analysis: Optimisation of protein precipitation conditions

3.1.1. Pilot study and selection of optimum extraction conditions

When added to media containing proteins, organic reagents act by decreasing the dielectric constant of the proteins of the medium, a. increasing electrostatic interactions between them and b. displacing water molecules around their hydrophobic areas. Thus, their solubility in the medium decreases, leading to aggregation and protein precipitation [21].

All four organic reagents used (MeOH, EtOH, ACN, acetone) gave acceptable recovery values (> 69.5%) for the six model compounds in the pilot study with clear supernatants for drugs' analysis in the HPLC after filtration (Figure 1). The only exception was EtOH when used as a precipitation reagent for danazol (Figure 1), which resulted in poor peak shape in HPLC despite the dilutions made.

Acidic reagents act by forming insoluble salts with the positively charged amino acids of a milk-based medium at pH below their isoelectric point [21]. The use of weak acids as protein precipitation reagents may be challenging for drugs demonstrating instability in acidic conditions. Hydrochloric acid, trichloroacetic acid and trifluoroacetic acid were particularly effective as precipitation reagents giving high recovery values (92.4–106.7%) for all ratios of the hydrophilic (metformin, metoprolol) drugs (Figure 1). They were not able to recover high amounts of the two most lipophilic drugs (danazol, itraconazole) from the medium though,

with 2.4 ± 0.1% maximum recovery at a 1:5 ratio for danazol and 0% recovery for all ratios for itraconazole achieved. As expected, a reason for the poor recoveries of lipophilic compounds in acidic reagents is their lower aqueous solubility, which is a barrier for the extraction potential of compounds of similar lipophilicity. The two weak acids (nifedipine, furosemide) were partially recovered using acidic reagents with the recovery percentage ameliorating by decreasing the FeSSGF:reagent ratio. The two weak bases (metformin, metoprolol tartrate) were almost 100% recovered at all ratios (Figure 1).

A three-way analysis of variance showed that the selection of protein precipitation reagent affected the % recovery values for all six drugs of the pilot study ($p < 0.05$) (Figure 2). Acetonitrile was proven the most effective (higher mean absolute recovery) for three out of six drugs, acetone for two and ethanol for one compound respectively in terms of mean absolute recovery. The presence of NaCl in the medium can increase drug recovery when acetone is used as a protein precipitation reagent. Crowell et al. [74] demonstrated that in acetone concentrations between 50 and 80% of the total mixture, NaCl concentrations > 10 mM in the medium led to protein % recovery values close to 100% for a number of proteins, such as α-casein, β-lactoglubulin and bovine serum albumin which are present in milk [75]. The effective entrapment of proteins in the precipitate possibly led to an increased amount of free drug available in the supernatant, resulting in higher recovery. Differences in efficiency among precipitation reagents can be attributed to the remaining proteins in the supernatant; since protein precipitation can only remove the larger proteins, leaving small proteins and peptides behind. These may interfere with the compounds of interest and have unpredictable effects (such as unexpectedly low drug recoveries) on drug quantification [21].

The effect of the FeSSGF:precipitation reagent ratio was evaluated in the pilot study for achieving maximum absolute drug recovery and adequate method sensitivity. Decreasing the medium:precipitation reagent ratio (from 1:1 to 1:5) did not show profound differences in

drug absolute recovery as far as organic reagents were concerned. With the exception of itraconazole, for which the % absolute recovery increased from approximately 78% to 99.9-106.6% when decreasing the FeSSGF:organic reagent ratio from 1:1 to 1:2, 1:3 and 1:5, all organic reagents resulted in % absolute recovery > 80% at all ratios used (Figure 2). A higher amount of organic solvent may increase the percentage of drug recovered by reducing solvation of the proteins in the aqueous medium, causing their precipitation. The statistical analysis showed that the ratio in which the precipitation reagent was added was statistically important. Reported p values for 4/6 drugs used in the pilot study were < 0.05 with the recoveries of metoprolol tartrate and danazol not being affected ($p = 0.86$ and 0.66 respectively) by the amount of precipitation reagent added (Figure 2). For the other drugs, 3 or 5 parts of protein precipitation reagent added in 1 part of FeSSGF resulted in higher % drug recovery than 1 part of reagent added to 1 part of FeSSGF prior to vortexing and centrifugation (Figure 1; red parts of the contour plot). Even though the differences among the protocols with different ratios were statistically significant, the difference may not always be practically important, as in most cases the method efficiency threshold set for the study (50 % absolute recovery) was met. Nifedipine is given as an example; the addition of 1 part of methanol in 1 part of FeSSGF, recovered approximately 101% of the drug, while addition of 5 parts recovered approximately 107% (Figures 1, 2). The same protocol by using 10% w/v TFA resulted in 26% and 72% values respectively. It is obvious that in the first case selection of a 1:5 ratio would not improve the extraction method but it would result in a loss of sensitivity, due to a bigger dilution of the medium with methanol. In the second case though, the difference is notably important and therefore for a compromise, % recovery, desired method sensitivity and HPLC method compatibility with the medium have to be considered.

Drug concentration had an effect on the percentage recovered using organic or aqueous solvents for protein precipitation. For the hydrophilic base (metformin), the % recovery values

were not affected by the drug concentration ($p > 0.05$) (Figure 2). In all other drugs of the pilot study, drug at high concentration was more effectively recovered ($p < 0.05$). Similarly to the example given above for the effect of protein precipitation ratio, the average difference between recoveries of "high" and "low" concentrations as given by the post-hoc Bonferroni test lied within a range between 0.5 and 12.3% in the range of drugs studied, with the highest recovery observed for "high" concentrations. Despite the slight differences in absolute recovery between concentrations, the method can still be used for drug analysis if the 50% absolute recovery limit is met and linearity is proven in the working concentration range. Reagents added at a 1:3 or 1:5 ratio to FeSSGF often dilute the sample significantly, driving its recovery below the LOQ of the method for the "low" concentration (Figure 2).

The reagents (two organic and one aqueous) selected were methanol, acetonitrile and 10% w/v trichloroacetic acid at a 1:2 FeSSGF: reagent ratio with the rationale of selection explained in the *Methods* part. Since the pilot study proved that the volume of reagent added for protein precipitation was statistically important, a relatively high reagent: FeSSGF ratio was selected (1:2), so that high % recovery and adequate method sensitivity could be maintained. For the final study and assessment for the rest of the model compounds, the highest of the two concentrations, which gave higher % recovery values in the pilot study was chosen.

3.1.2. Main study and selected protocol application

The two organic reagents used, methanol and acetonitrile, added at a 1:2 FeSSGF:reagent ratio, gave high recovery values for all the compounds over a wide range of lipophilicity (log P = -0.56-8.81) and ionisation with the exceptions of atovaquone and MK-C4. Atovaquone (log P = 5.07) was only recovered by 33.4% at 25 μg/mL in FeSSGF when methanol was used, while acetonitrile recovered 82.2% of the same drug concentration (Figure 3). For drug extraction using acetonitrile, atovaquone studies in plasma [76] and whole blood [77] gave similar results to our study. For methanol, since atovaquone's solubility in it is much higher than the concentration used, a possible reason of the significantly low recovery values could be the loss of analyte due to its occlusion in the precipitate [78]. The drug's extremely high affinity for plasma proteins (> 99.5% bound) [79] and its high affinity to fat, as described in *in vivo* studies which showed increased drug bioavailability after co-administration with a high fat meal [80], could indicate a strong interaction with fat or proteins of the fed state medium. This interaction may have not been disrupted by the application of methanol, with the drug being entrapped in the precipitate.

The aqueous reagent (10% w/v TCA) added at the ratio mentioned (1:2) was proven effective only for highly soluble (aqueous solubility > 100 μg/mL) APIs with log P values < 2 with the % recovery of weak bases (metformin hydrochloride, metoprolol tartrate, atenolol, paracetamol), approaching 100% (Figure 3). The above compounds were mostly unionised at pH 5, but were likely negatively ionised at the low pH of the acidic supernatant. Precipitation with trichloroacetic acid gave poor recovery values (0%) for atovaquone, because the drug as a lipophilic weak acid co- precipitated with the proteins [81]. The same applied for MK-C4 (log P = 8.81), which is an extremely lipophilic drug with high affinity for adipose tissue [82].

% recovery values lower than 100% when organic reagents were used for protein precipitation were probably not attributable to the interaction of the drug with protein molecules of the supernatant, but due to its entrapment in the precipitate. This hypothesis was confirmed by superimposing the spectra of the supernatant and drug standards in an acetate buffer:precipitation reagent mixture. The drugs selected demonstrated variable % recovery values in the three reagents selected (methanol, acetonitrile, 10% w/v TCA). Nevertheless, the spectra were identical in all cases despite high or low % drug recovery values and no other interference was observed in the peak of the drug. Moreover, the retention time of all drugs in the chromatogram remained constant, which implied that there was no change in the structure of the drug molecule (data not shown). Therefore, the results indicated that the drug quantified was the free drug in solution without any interference from the biological matrix.

The % recovery obtained for all drugs was below the 15% threshold set for the coefficient of variation (Figure 4), with samples extracted using TCA demonstrating the highest variability, but still within the proposed limits.

3.1.3. Prediction of the effect of physicochemical properties on extraction protocol (PP) selection

The variables and their interactions of the PLS models examined are summarized in Figure 5. The PLS models constructed for % recovery values when MeOH, ACN and 10% w/v TCA were used as protein precipitation reagents were defined by 1, 1 and 2 Principal Components respectively. The PLS model developed for 10% w/v TCA was a good fit to the experimental values (R^2 = 0.87) and showed good predictive power (Q^2 = 0.83). The models developed for MeOH and ACN can only account for a very low percent of Y variability (R^2 = 0.34 and 0.23 respectively), and have limited predictive power (Q^2 = 0.24 and 0.05 respectively), according to the threshold (Q^2 = 0.5) set for the study.

The model demonstrated that lipophilicity (log P) is defined as negative predictor for % drug recovery in all three cases when MeOH, ACN and 10% w/v TCA were used as protein precipitation reagents in FeSSGF treatment with its effect in the cases of MeOH and TCA 10% w/v being statistically significant, as indicated by the high VIP factor (Figure 5). A higher partition coefficient indicates higher tendency of the solutes distribution to the lipid phase of the medium [83], which could be limiting the extraction potential of the reagent.

In cases where methanol was selected as protein precipitation solvent, drug lipophilicity affected the extraction of compounds regardless of their ionisation state, with a bigger effect on neutral and acidic compounds (VIP > 1), and compounds being in the unionised state in the working pH, as indicated by the negative standardised coefficients of log P interactions with the properties mentioned. Drug distribution in the lipid medium fraction is facilitated for unionised drugs, as ionised molecules have to dispose a part of their hydration water in order to permeate the lipid bilayer, a process energetically unfavourable [84]. The above observations of the drug physicochemical properties which affect extraction from FeSSGF using methanol, denote that even though the effect of ionisation percentage does not have a significant impact by itself, it can have a negative effect on the amount recovered in lipophilic drugs.

For the use of 10% w/v TCA in protein precipitation, the main factors governing the % recovery are the drug's log P, drug bound protein fraction (negative effect) and its aqueous solubility (positive effect). Even though the exact mechanism of protein precipitation is not fully understood, a proposed mechanism of action suggests the segregation of the protein bound water, with the type of the proteins not affecting the method efficiency, which is also acid-concentration dependent [85]. The concentration of TCA used in the current study may only be adequate to precipitate a portion of proteins, with the drugs highly bound to proteins being trapped in the precipitate. High lipophilicity and high aqueous solubility as negative and positive predictors respectively may be explained by the aqueous nature of the precipitation

reagent. Moreover, the fact that the interactions of drug protein bound fraction with log P and aqueous solubility have a negative effect on drug recovery, strengthens our hypothesis that the inability of the TCA to break the drug-protein interactions under the stated experimental conditions is an unfavourable factor, even for the extraction of water soluble drugs. Similarly to what was observed in the pilot study, PLS regression showed that TCA is a suitable reagent for highly soluble weak bases (positive log aq sol*base interaction, Figure 5), while it affects the extraction of lipophilic bases or bases which are unionised in the medium's pH in a negative manner, which was demonstrated by the negative log P*base and union fr*base negative standardized coefficients (Figure 5).

The lack of the model's predictive ability when ACN was used as a PP reagent can be explained by the reagent's extremely high extraction ability in the whole range of drugs (> 90% recovery for 17/20 drugs studied) and low variance, with most values falling close to 100%.

3.1.4. Designing a roadmap for effective sample treatment using protein precipitation

In summary, MeOH and ACN can be effective for drugs exhibiting a wide range of lipophilicity and the use of one of the other is usually effective for drugs of log P values of -0.5 to 5 (Figure 6). For hydrophilic to moderately lipophilic bases (metformin, metroprolol tartrate, log P < 2), the use of 10% w/v TCA was preferred over the two organic solvents, due to a better peak shape in the HPLC analysis. According to the findings of the PLS regression analysis, TCA is the most efficient reagent (higher absolute % recovery) for highly soluble drugs and drugs which exhibit a low affinity for proteins. Drugs of moderate lipophilicity were equally well recovered using either MeOH or ACN, therefore, both reagents could be used for the extraction of drugs of log P between 2 and 5. Issues with low % recovery with the use of MeOH were only encountered with some drugs of extreme lipophilicity (log P > 5), for which ACN was the most effective option (atovaquone, MK-C4). Consequently, ACN would be the

522 best choice of the three reagents (Figure 6), as drugs at this log P range were in all cases at
523 more than 80%, while the use of MeOH and 10% w/v TCA could give absolute recoveries as
524 low as 24 and 0% respectively. Therefore, taking the method's high recovery and low
525 variability obtained into consideration, we suggest that protein precipitation may be used in
526 biorelevant dissolution and solubility studies with the use of a single correction factor based
527 on the drug % recovery obtained, when a single calibration standard in FeSSGF is used. For
528 the accurate determination of a dissolution profile the analyst should consider using at least
529 one second calibration point for the quantification of drug released in the initial time points.
530 As demonstrated in the pilot study, drug recovery is concentration dependent. Calibration
531 standards prepared in triplicate would be required for the low concentration, in order to
532 compensate for the higher variability of the method.

533 **3.2. Drug analysis: Optimisation of solid phase extraction conditions**

534 ***3.2.1. Pilot study and effect of elution volume***

535 The results of the pilot study, where tC_{18} cartridge was selected as a starting point are
536 presented in Figure 7b. It can be seen that the specific cartridge can be effectively used for a
537 range of compounds from moderately polar to non-polar. Drugs of log P between 0.74 and 4.2
538 were recovered at a percentage higher than 60% (Figure 7b). Even though the minimum elution
539 solvent (2 mL) is higher than two bed volumes (500 mg sorbent = 600 μL bed volume) which
540 is required for effective extraction [21], it was shown that elution with 5 mL increased the %
541 recovery values by a significant amount ($p < 0.05$) compared to 2 mL for all compounds of the
542 pilot study (from 6.5% increase for nifedipine to 1700 % increase for danazol) (Figure 7b).
543 Therefore, 5 mL was selected as the elution volume to proceed with the rest of the model
544 compounds.

3.2.2. Effect of cartridge

The results of the complete study where the cartridges and elution conditions selected were applied for all of the model compounds are presented in Figure 7a. tC_{18} cartridges can generally be used for a range of compounds from moderately polar to non-polar. Non-polar parts of the analyte develop Van der Waals interactions with the C_{18} non-polar groups of the sorbent, leading to selective retention of the analyte of interest, before its elution with an appropriate elution solvent. HLB was another type of sorbent used in this study and is a co-polymer comprised of two different monomers; one hydrophilic (N-vinylpyrrolidone) and one hydrophobic (divinylbenzene). The use of HLB cartridges has also been found to be effective for both polar and non-polar compounds [86].

For compounds of log P values between 1.95 and 4, there was no clear pattern as to which should be selected in favour of the other for optimum % recovery values using log P as a selection criterion. Nevertheless, the use of one or the other cartridge (tC_{18} or HLB) in compounds of moderate lipophilicity (log P 2-4) recovered a minimum value of 69.5% of the initial compounds in FeSSGF for the optimum protocol at each case (Figure 7a). MK-C1, a compound on the verge of the threshold set for moderate lipophilicity (log P = 4) was poorly recovered in all cases 1.76 ± 0.33% and 0.15 ± 0.0 %for tC_{18} and HLB cartridges respectively. Its recovery was not improved despite protocol modification (tC_{18} and elution with MeOH, recovery = 8.80 ± 0.61).

For most hydrophilic and lipophilic compounds, HLB was proven more effective, as it increased the % recovery values of the compounds which could not be effectively extracted (< 15% absolute recovery) using tC_{18} cartridges (metformin hydrochloride, paracetamol, atovaquone, itraconazole, MK-C1, MK-C2, MK-C4), but not always to a great extent. For the drugs which could not be effectively extracted with the protocol used with tC_{18} cartridges, a

switch to HLB achieved a meaningful improvement in extraction performance only for paracetamol and MK-C2 (Figure 7a).

SPE was incompatible with the extremely lipophilic model drugs studied. Using the current protocols, the recoveries of drugs of extreme lipophilicity (log P > 5) were higher with the use of HLB cartridges but still relatively low (5.7 ± 0.2%, 27.9 ± 0.7%, 22.1 ± 3.5% and 0% against 1.9 ± 0.1%, 0 %, 15.3 ± 0.2% and 0% for tC_{18}, for atovaquone, itraconazole, lapatinib, and MK-C4 respectively (Figure 7a). Previous studies with extraction of itraconazole with HLB cartridges from biological matrices demonstrated higher recovery values than the ones presented in this study. Although HLB cartridges have been more successfully used for extraction of itraconazole, these studies were in blood, [87] plasma [88] and surface waters [89] and the cartridges could be incompatible with the fed state medium used. The modification proposed in the methodology did not improve the recovery of the drug.The low recoveries of the extremely lipophilic compounds and the lack of pattern in terms of cartridge selection for the extraction of the moderately lipophilic compounds could indicate that the critical parameter for SPE optimisation is not the log P value of an API, but the type of interactions it develops with components (lipids, proteins) of the milk-based matrix. For atovaquone, 5 mL MeOH were also tested with tC_{18} cartridge giving somewhat better results but still low recovery values (17.5 ± 0.6 %) (Figure 8). The poor SPE recovery values for the specific drug, along with the low % recovery when MeOH was used in protein precipitation, supports the initial hypothesis that strong interaction with components of the fed state medium could be the main obstacle which has to be surpassed for effective extraction. In the cases of lapatinib, MK-C1, MK-C2, MK-C3 and MK-C4, 100% methanol was used as elution solvent in order to increase the protocol efficiency with tC_{18} cartridges with the stronger elution volume improving the percentage of drug eluted significantly in all drugs apart from MK-C3. Drug analysis results

593 for which modification of the SPE protocol led to an increase in % recovery are presented in
594 Figure 8.

595 For hydrophilic drugs (log P < 2), significant difficulties in effective drug recovery
596 were encountered only in the case of metformin. Metformin is an extremely polar molecule
597 which lacks hydrophobic functional groups. It was suggested that, due to the molecule's
598 polarity, retention on the cartridge's hydrophobic functional groups was poor. While SDS
599 conditioning did improve the recovery of metformin, the amount of drug recovered was still
600 low (< 10%, data not shown). The most effective strategy in the case of metformin was the
601 omission of the washing step. The omission of the washing step (tC_{18} and HLB cartridges),
602 which improved the % recovery significantly (≈ 34% and 20% respectively-data not shown),
603 was a far more effective strategy. Its combination with a change to a more hydrophilic cartridge
604 like $C_{8,}$ a recovery value of 49.6 ± 1.9%, just below the acceptable recovery limit, was achieved
605 (Figure 8). The functionalization of its silanol groups comprises of chains of eight carbon
606 molecules instead of the eighteen like in tC_{18}, therefore it is suggested that the drug is retained
607 to the column via weaker hydrophobic interactions with the stationary phase's silanol groups
608 which are easier to break.

609 Our observations for metformin and itraconazole (extremely hydrophilic and extremely
610 lipophilic compounds respectively) (Figures 7a,b) were in agreement with previous studies
611 which suggested that C_{18} cartridges are often a poor choice for drugs of extreme or poor
612 hydrophilicity. Metformin [90] and itraconazole [91] were recovered by < 20% and < 40%
613 respectively when eluted from C_{18} cartridges using methanolic solutions (studies in aqueous
614 solutions and human liver microsomal' fraction respectively). Recoveries using the two
615 different cartridges were significantly different in the majority of cases, ($p < 0.05$, 16/20 drugs),
616 in the range of model compounds studied (Figure 7a), meaning that selecting one over the other
617 can have a significant impact on the amount of drug to be recovered using a specific protocol

618 in a study. Sample variability obtained with solid phase extraction was significantly higher than
619 the variability observed using the protein precipitation protocols developed. For five of the
620 drugs tested (metoprolol tartrate, pravastatin sodium, celecoxib, MK-C1 and lapatinib) the %
621 CV obtained was above the variability threshold set for the study (Figure 4). No apparent
622 differences were observed between the different cartridges or trends related with drug
623 lipophilicity.

624 ***3.2.3. Prediction of the effect of physicochemical properties on extraction protocol (SPE)***
625 ***selection***

626 The variables and their interactions of the PLS models examined are summarized in
627 Figure 9. The PLS models constructed for % recovery values when tC_{18} and HLB cartridges
628 were used (standard protocol) were defined by 1 and 3 Principal Components respectively. The
629 PLS model developed for HLB was a good fit to the experimental values ($R^2 = 0.87$) and
630 showed good predictive power ($Q^2 = 0.83$), while the model developed for the SPE extraction
631 using a tC_{18} cartridge can only account for a low percent of Y variability ($R^2 = 0.34$), and has
632 poor predictive power ($Q^2 = 0.24$). In both cases, the parameter having the most prominent
633 positive effect was the log P*log aqueous solubility interaction (Figure 9), which is attributed
634 to retention of a higher amount of drug in the SPE cartridge during the initial loading step, and
635 also a more effective elution using a polar MeOH or MeOH/H_2O solvent in the elution step. In
636 tC_{18}, drug lipophilicity alone, but also its interactions with basic and neutral compounds,
637 affected drug extraction negatively. The same applied for compounds which act as ampholytes
638 in an aqueous environment while acidic compounds were easier to extract (positive
639 standardised coefficient for weakly acidic compounds, VIP > 1). The recovery dependence of
640 the compounds' ionisation state can be attributed to the presence of ionic interactions between
641 charged drugs with the residual silanol groups of the cartridge which are unable to break with
642 unbuffered MeOH:H_2O elution solvents [92]. Similar conclusions were deducted from the PLS

643 regression model for HLB, with the difference being the negative correlation between log P
644 and extraction efficiency, which was only present for unionised drugs in the working pH (log
645 P*union fr interaction, Figure 9). The positive effect of aqueous solubility and its interaction
646 with log P can be attributed to the more effective elution of polar compounds, when eluted with
647 MeOH.

648 ***3.2.4. Designing a roadmap for effective sample treatment using solid phase extraction***

649 In summary, tC_{18} and HLB cartridges can be effectively used for drugs of low to
650 intermediate lipophilicity (log P = 0-5) while for extremely hydrophilic compounds, the use of
651 C_8 cartridge and the omission of the washing step (where possible) were the most effective
652 options (Figure 10). In the whole range of compounds, it was shown that both HLB and tC_{18}
653 cartridges can be used, with HLB being more efficient for highly soluble drugs and also for
654 weak acids, which are fully unionised at the working pH. Therefore, for highly soluble or
655 weakly acidic compounds with a log P value between 0 and 5 the use of HLB cartridges is
656 suggested. For drugs of extreme lipophilicity (log P > 5), increasing the strength of the elution
657 solvent to 100% organic content usually increased the amount of drug recovered, but in certain
658 cases recovery did not exceed 10-20% despite the attempted modifications of the initial
659 protocol. Taking that into consideration, we suggest the use of 100% MeOH as elution solvent
660 for compounds of extreme lipophilicity (using either one of the cartridges). HLB is suggested
661 as the SPE cartridge of choice for the same reason as in compounds of moderate lipophilicity.
662 For extremely lipophilic compounds (log P < 0), alternative cartridges could possibly be used
663 (e.g. C_8) for maximum efficiency, and if the medium permits, the washing step after sample
664 loading in order could be omitted so as to maximise the amount of compound still retained on
665 the cartridge before the elution step. Due to the high variability and poor recovery results
666 obtained for many of the drugs across the range of lipophilicity studied, we have concluded
667 that solid phase extraction, used as is, without a prior purification step is not an adequately

robust technique for sample clean-up. We suggest the use of a correction factor by using at least a triplicate for each standard in FeSSGF across the range of concentrations used in biorelevant dissolution and solubility studies in order to account for the high method variability.

4. Conclusion

Prediction of gastric food effect on drug absorption has been a big challenge for the pharmaceutical industry. Even though the *in vivo* properties of the fed state gastric environment have been quite well determined and some progress has been made with the development of gastric biorelevant media, a universal robust predictive analytical method has not been yet developed. The utility of such a method will allow the effective extraction and drug quantification of a range of drugs in heterogeneous fed biorelevant media selected on the basis of properties related to the medium, active ingredient or both. The above would drive drug analysis towards more standardised protocols and away from the current drug-by-drug assessment for optimal treatment conditions. The current study assessed the effective quantification of drugs, based on their physicochemical properties from milk-based media using two extraction techniques: i. Protein precipitation and ii. Solid phase extraction. The current study demonstrated that the use of three precipitation reagents (methanol, acetonitrile and 10% w/v trichloroacetic acid) at a FeSSGF:reagent ratio of 1:2, when used according to the guidelines proposed, provided a simple sample preparation method which can be decided based on drugs' selected physicochemical properties. 10 % w/v trichloroacetic acid was mostly suitable for weak bases of log P < 2, while either methanol or acetonitrile were effective for all the other model drugs. It has also been shown that the solid phase extraction protocols proposed using three different cartridges (tC_{18}, C_8 and HLB) provided good sample treatment methods for all drugs of a wide range of log P values (0.30–4) achieving recovery values > 69.5%.

692 Modifications of the initial protocols, involving cartridge treatment and different elution
693 solvents, improved the % recovery of the extremely lipophilic and extremely hydrophilic model
694 drugs (9-60%), but with results still indicating that solid phase extraction is possibly not the
695 method of choice for drugs of higher lipophilicity. Knowledge of the drug's key
696 physicochemical properties is critical for the selection of the optimum extraction protocol for
697 milk-based fed state media. In this study, the effect of the drug's physicochemical properties
698 (lipophilicity, ionisation, aqueous solubility, protein affinity) and their interactions on recovery
699 efficiency from fed state media were assessed, allowing the selection of the optimum extraction
700 tool for drug quantitative analysis. The roadmaps developed for the two extraction techniques,
701 can provide a starting point towards the development of a unified guideline, where selection of
702 the extraction method can be made on the drug physicochemical profile, where drug
703 physicochemical properties may be used to estimate the approximate % recovery in a specific
704 extraction. It is evident though that the findings of the current study have been based on the
705 effect of the physicochemical properties of the drug substance and may not always be directly
706 applicable in the analysis of formulations, where drug-excipient or medium-excipient
707 interactions may take place. Further studies are required for the elucidation of the analytical
708 profile of a range of compounds in heterogeneous biorelevant media simulating the gastric fed
709 state, while the effect of excipients in drug analysis needs to be assessed in order to determine
710 the method's applicability in biorelevant dissolution studies.

711

712

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6. References

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Tables

Table 1 Physicochemical properties and working concentrations of model compounds.

Drug	**log aqueous solubility (mg/ mL) [25-29]**	**log P [26, 28-41]**	**pKa [28, 39, 42-50]**	**Working concentrations (µg/ mL)** (d)	**Plasma protein bound** (b)
Metformin hydrochloride	2.48	-0.56	12.40	2000/2	0.035
Atenolol	1.11 (a)	0.23	9.60	200	0.129
Paracetamol	1.24 (a)	0.30	9.50	200	0.124
Furosemide	-1 (b)	0.74	3.90	80/1	0.031
Metoprolol tartrate	1.01	1.95	9.70	200/10	0.244
Pravastatin sodium	-0.42 (b)	2.20	4.36	40	0.767
Nifedipine	-1.90	2.91	3.93	60/1	0.999
Propafenone hydrochloride	-0.82 (b)	3.39	9.27	600	0.957
Celecoxib	-2.52 (b)	3.47	11.10	100	0.975
Ketoconazole	-2.57 (a)	3.72	3.25, 6.22	150	0.986
MK-C1	-2.53 (c)	4 (c)	6.5 (c)	35	0.908
Azithromycin	3 (a)	4.02	8.74, 9.45	1000	0.558

Danazol	-3	4.20	none (b)	25/1	0.983
Atorvastatin calcium	-2.59 (a)	4.22	4.46	160	0.178
Atovaquone	-3.37	5.07	5.01 (b)	25	0.995
MK-C2	-3 (c)	5.11 (b)	4.48, 5.74 (c)	1300	0.985
Itraconazole	-6	6.20	3.70	0.5/0.1	0.997
Lapatinib	-5.68 (a)	6.30 (a)	6.34 (a)	8.7	0.998
MK-C3	insoluble (c)	6.31 (b)	3.53 (c)	500	0.997
MK-C4	-4 (c)	8.81 (c)	none (c)	4.5	0.999

(a) Sci-Finder

(b) Calculated using Advanced Chemistry Development (ACD/Labs) Software V11.02 (© 1994-2016 ACD/Labs)

(c) Data provided by Merck and Co, INC

(d) "High" concentration/"Low" concentration. "High" concentration = $\frac{drug\ dose\ (mg)}{500\ (mL)(volume\ typically\ used\ in\ gastric\ fed\ dissolution\ studies)}$ or solubility in mik/FeSSGF (literature values) or performed solubility study (24 h) in FeSSGF. "Low" concentration = 10 x LOQ in acetate buffer, MeOH:buffer (1:1) or ACN:buffer (1:1) and ≤ 0.2 x "high" concentration. Otherwise, 0.2 x "high" concentration was selected.

999 **Table 2** HPLC methods (or modification of published methods) used for the quantification of the model compounds.

Drug	Column	Mobile phase	Flow rate (mL/min)	Temperature (° C)	Inj. volume (µL)	UV detection (nm)
Nifedipine [53]	Thermo Hypersil BDS C_{18}, 300Å, 250 x 4.6 mm, 5 µm	MeOH:H_2O 60:40	1	20	50	238
Furosemide [54]	Thermo Hypersil BDS C_{18}, 300Å, 250 x 4.6 mm, 5 µm	MeOH:Formic acid 0.1% v/v 60:40	0.8	25	20	233
Metoprolol tartrate [55]	Thermo Hypersil BDS C_{18}, 300Å, 250 x 4.6 mm, 5 µm	MeOH:TFA 0.1% v/v 47:53	0.8	10	50	274
Danazol [56]	Thermo Hypersil BDS C_{18}, 300Å, 250 x 4.6 mm, 5 µm	MeOH:H_2O 85:15	1	25	100	285
Metformin hydrochloride [57]	Vydac Diphenyl, 300Å, 250 x 4.6 mm, 5 µm	ACN:Phosphate buffer 0.02 M (pH = 7) 70:30	1	20	20	236
Itraconazole [58]	Thermo Hypersil BDS C_{18}, 300Å, 250 x 4.6 mm, 5 µm	MeOH:H_2O 80:20	1	35	100	260
Celecoxib [59]	Thermo Hypersil BDS C_{18}, 300Å, 250 x 4.6 mm, 5 µm	MeOH:H_2O 70:30	0.8	25	100	251

Atovaquone **[60]**	Waters Spherisorb S5 ODS2, C_{18}, 80Å, 250 x 4.6 mm, 5 µm	ACN:TFA 0.4% v/v 70:30	1.5	25	50	253
Paracetamol **[61]**	Thermo Hypersil BDS C_{18}, 300Å, 250 x 4.6 mm, 5 µm	MeOH:H_2O 20:80	1	10	20	257
Ketoconazole **[62]**	Thermo Hypersil BDS C_{18}, 300Å, 250 x 4.6 mm, 5 µm	MeOH:H_2O:DEA 75:25:0.1	1	25	50	260
Atenolol **[63]**	Thermo Hypersil BDS C_{18}, 300Å, 250 x 4.6 mm, 5 µm	MeOH:Phosphate buffer 0.01 M (pH = 4.5) 20:80	1	25	50	240
Azithromycin **[64]**	Waters Symmetry C_8, 100Å, 250 x 4.6 mm, 5 µm	MeOH:Phosphate buffer 0.3 M(pH = 7.5) 20:80	1.2	40	100	210
Pravastatin sodium **[65]**	Agilent Eclipse XDB C_{18}, 120Å, 250 x 4.6 mm, 5 µm	MeOH:Phosphate buffer 0.03 M (pH = 7) 55:45	1	25	100	238
Lapatinib **[66]**	Agilent EC-C_{18} Poroshell, 150 x 4.6 mm, 2.7 µm	ACN:Ammonium acetate 0.05 M (pH = 4.5) Gradient (0-5 min 40:60/ 5-13 min 58:42/ 13-17 min 90:10/ 17-19 min 40:60)	0.9	40	50	261

Propafenone hydrochloride **[67]**	Agilent Eclipse XDB C_{18}, 120Å, 250 x 4.6 mm, 5 µm	MeOH:ACN:TEA: H_2O 50:7.5:0.1: q.s 100 (pH= 2.9)	0.8	25	20	248
Atorvastatin calcium **[68]**	Thermo Hypersil BDS C_{18}, 300Å, 250 x 4.6 mm, 5 µm	ACN:Phosphate buffer 0.025 M (pH = 6) 40:60	1.5	30	50	246
MK-C1*	Waters Symmetry Shield C_{18}, 100Å, 50 x 4.6 mm, 5 µm	ACN:Phosphate buffer 0.025 M (pH = 2.5) Gradient (0-2 min 65:35/ 2-2.01 min 90:10/ 2.01-3 min 90:10/ 3-3.01 min 65:35)	3	40	20	214
MK-C2*	Phenomenex Onyx monolithic $C_{18,}$ 300Å, 100 x 4.6 mm	ACN: 0.1% H_3PO_4 70:30	5	40	10	240
MK-C3*	Agilent Prorochell C_{18}, 120 Å, 50 x 2.7 mm	ACN:Sodium Phosphate 0.005 M (pH = 7) Gradient (0-0.5 min 40:60/ 3-3.5 min 10:90/ 3.51-5 min 40:60	1	40	25	250
MK-C4*	Phenomenex Onyx monolithic $C_{18,}$ 300Å, 100 x 4.6 mm	ACN:H_2O 70:30	3.5	40	100	220

1000 *HPLC methods were provided by Merck and Co, INC.

Figure captions

Figure 1 Protein precipitation reagent-ratio-log P gradient map; contour plot of % recovery after protein precipitation of drug solution at "high" concentration in FeSSGF for the six compounds of the pilot study. "Warm" colours (red, orange) indicate high recovery values and "cold" colours (green, blue) indicate poor reagent performance. Log P values of -0.56, 0.74, 1.95, 2.91, 4.20, 6.20 correspond to metformin hydrochloride, furosemide, metoprolol tartrate, nifedipine, danazol and itraconazole, respectively.

Figure 2 Three-way ANOVA results of protein precipitation conditions for the six drugs of the pilot study. Graphs denote % drug recovery for all reagents at high (blue) and low (green) concentrations. The fractions presented in the centre of the Figure denote the FeSSGF:reagent ratio used in the protocol (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Bonferroni post-hoc test).

Figure 3 Mean % recovery values of the selected protein precipitation reagents (MeOH, ACN, 10% w/v TCA) for the 20 model drugs (Table 1), a. vs. log P and log aqueous solubility (mg/mL), b. vs. log P and pKa, c. vs unionized fraction and log P and d. vs charge vs protein bound fraction as 3D scatter plots.

Figure 4 % recovery variability obtained with the use of the selected protein precipitation reagents (MeOH, ACN, 10% w/v TCA) or SPE cartridges (tC_{18}, HLB) for the 20 model drugs (Table 1), expressed as % CV. The dashed line represents the acceptable limit proposed for mean % recovery.

Figure 5 Variable importance in the projection (VIP) plot with the variables classed according to their importance of the response (left) for the selected protein precipitation protocols. Standardised coefficients corresponding to the variables (and their interactions) studied. Green colour denotes coefficients of VIP values > 1, which are considered influential to the response value (right).

Figure 6 Roadmap of protein precipitation conditions selected for maximum % drug recovery from the fed gastric medium.

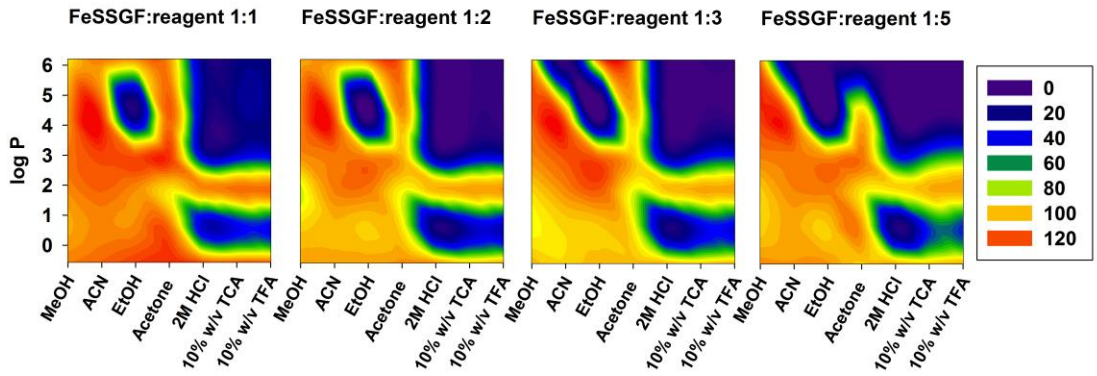
Figure 7 a. % recovery values of model drugs using the SPE protocols for tC_{18} and HLB cartridges. **b.** % recovery values of model drugs of pilot study using different elution volumes (tC_{18} SPE cartridge). Stars denote significant differences between % recoveries of **a.** different

cartridges and **b.** elution volumes (b) (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, two-sided t-test). The dashed line represents the acceptable limit proposed for mean % recovery.

Figure 8 % recovery values of model drugs, in cases where the SPE protocols had to be modified (< 50% recovery obtained with the initial analytical methodologies) The bar charts represent the drugs where modifications of the standard SPE protocols led to improved % recovery values.

Figure 9 Variable importance in the projection (VIP) plot with the variables classed according to their importance of the response (left) for the selected SPE protocols. Standardised coefficients corresponding to the variables (and their interactions) studied. Green colour denotes coefficients of VIP values > 1, which are considered influential to the response value (right).

Figure 10 Roadmap of solid phase extraction conditions selected for maximum % drug recovery from the fed gastric medium.



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1048 Figure 1

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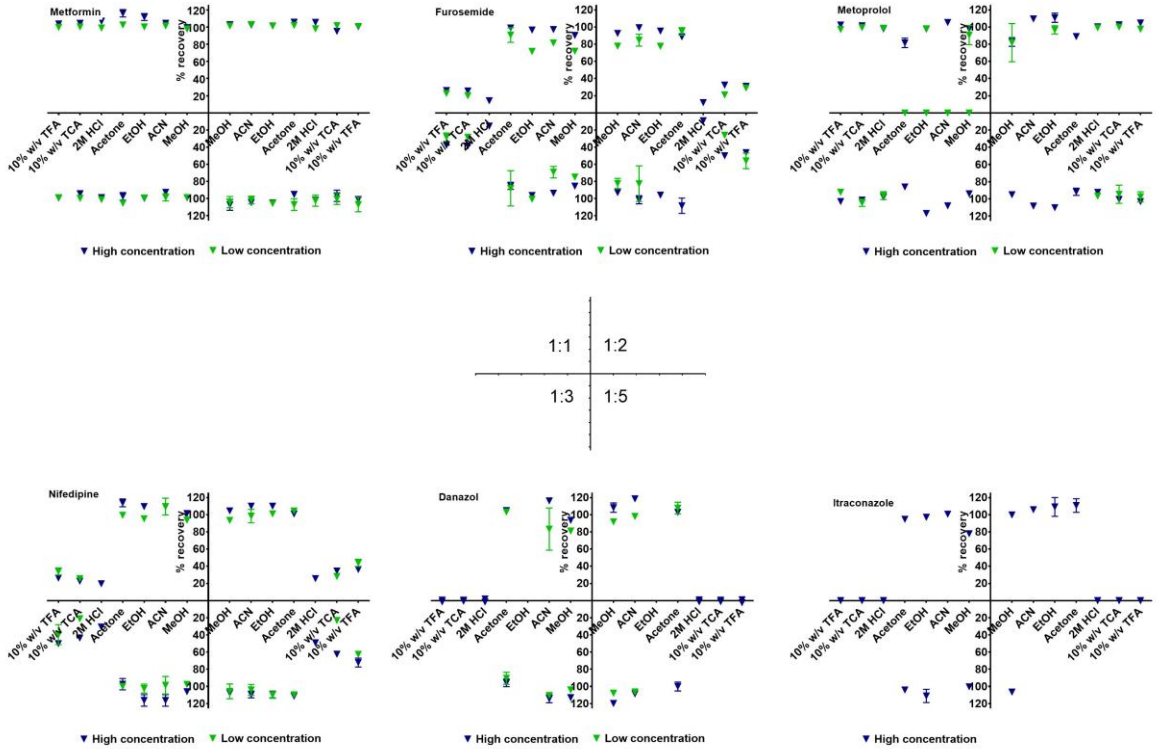
Metformin
Furosemide
Metoprolol
% recovery
10% w/v TFA
10% w/v TCA
2M HCl
Acetone
EtOH
ACN
MeOH
High concentration
Low concentration
1:1
1:2
1:3
1:5
Nifedipine
Danazol
Itraconazole

Figure 2

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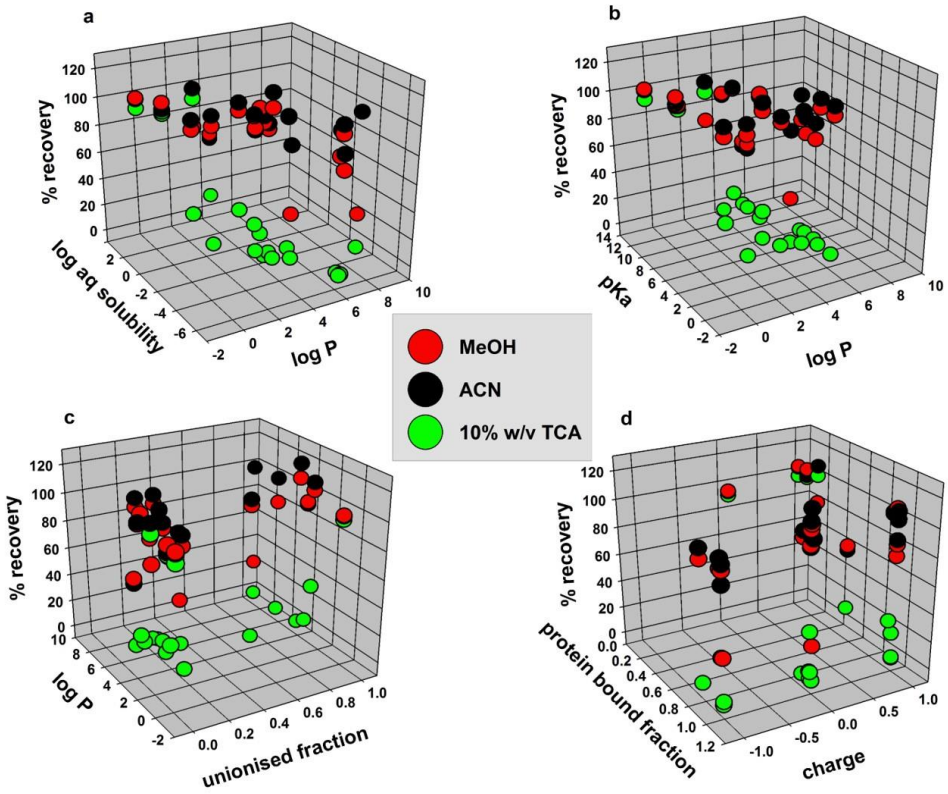

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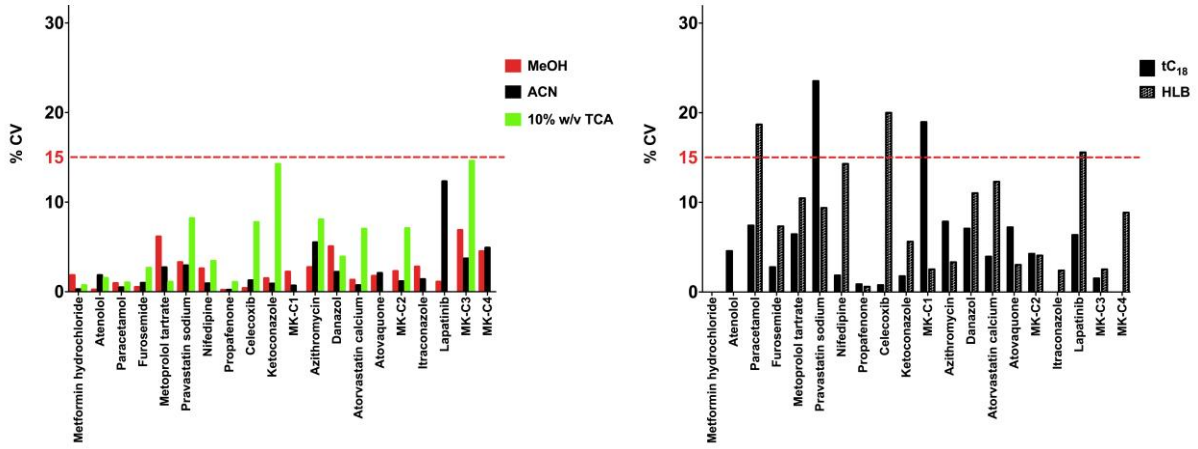

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1066 Figure 4

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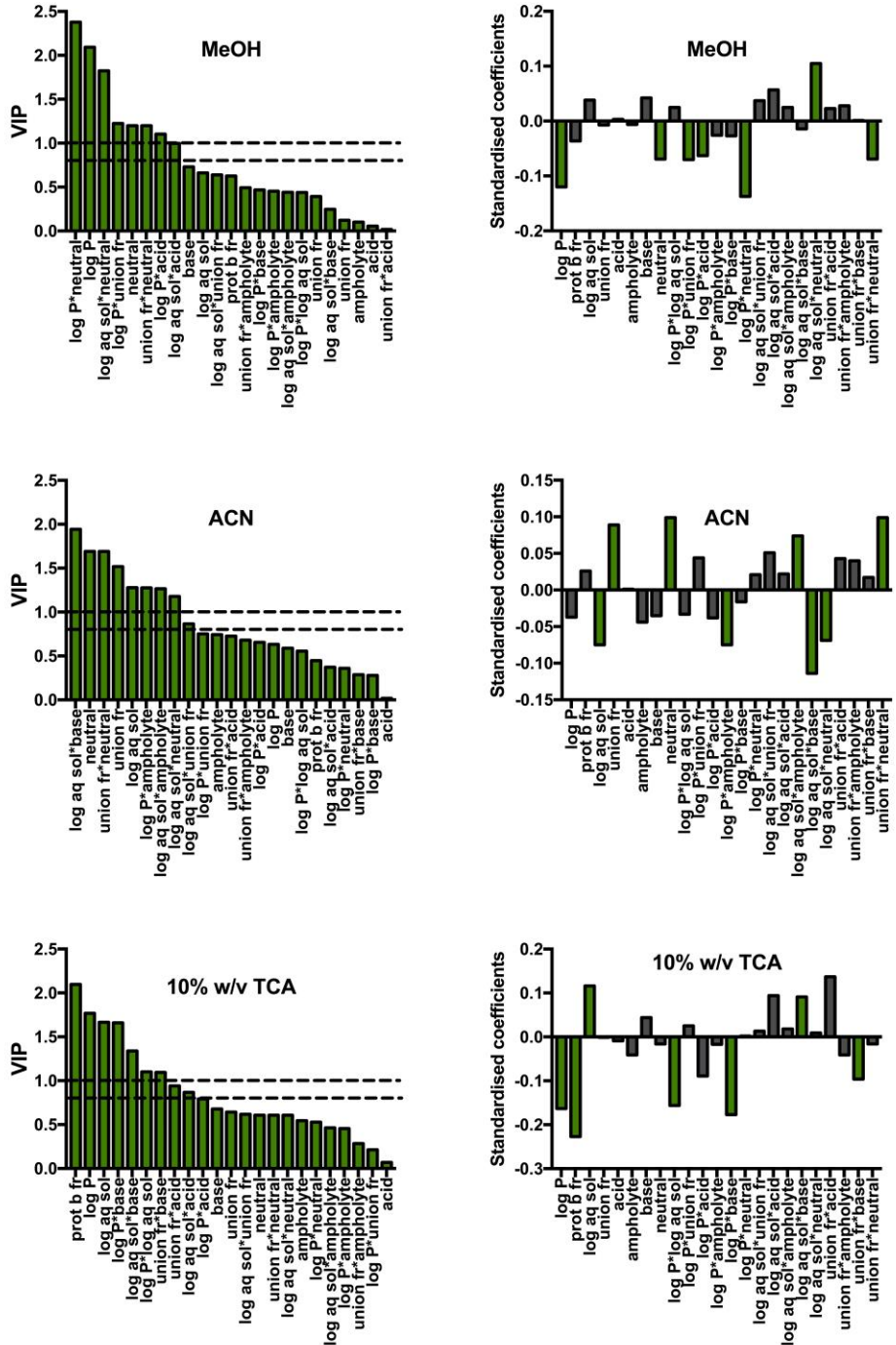

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1070 Figure 5

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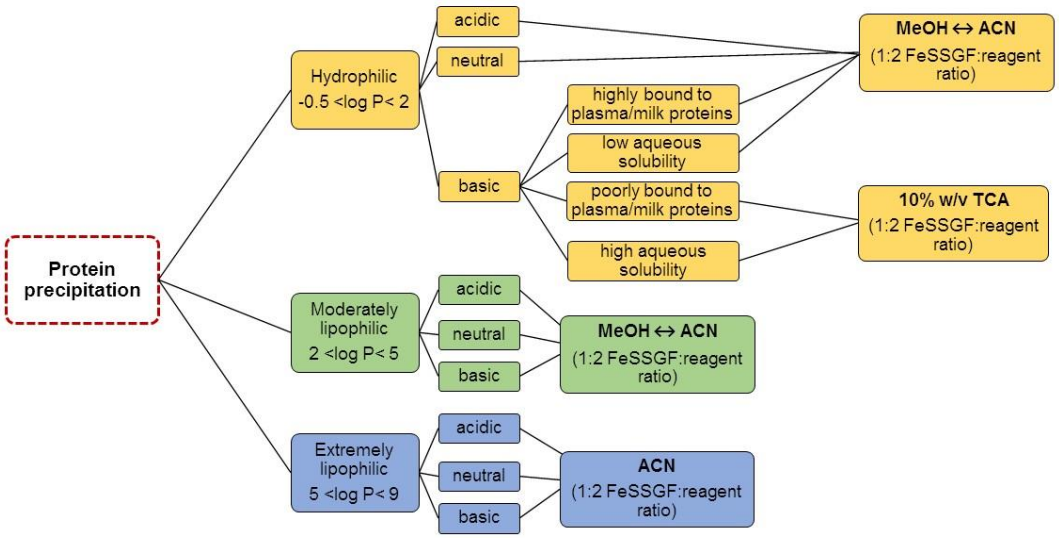

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1074 Figure 6

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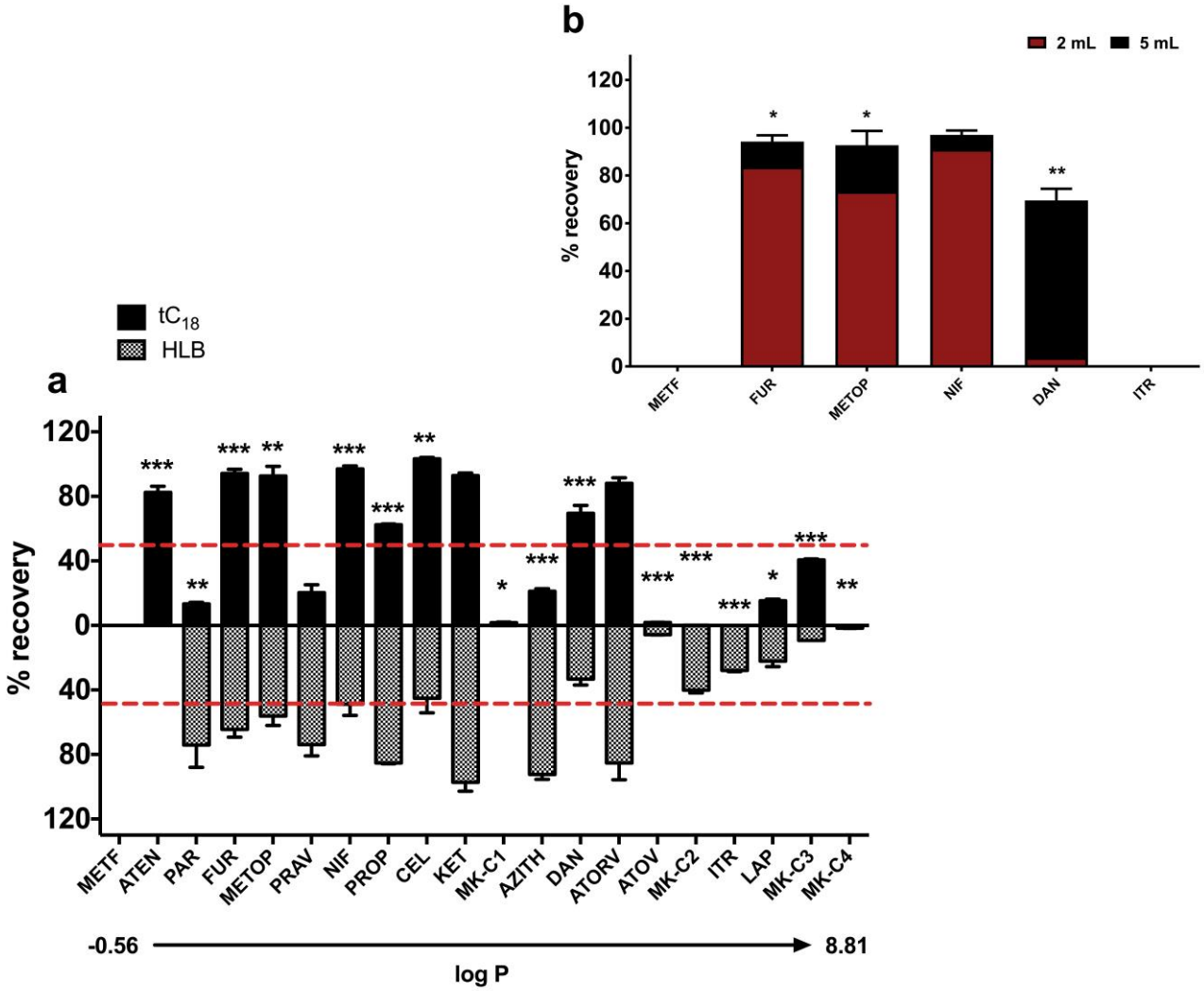

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1078 Figure 7

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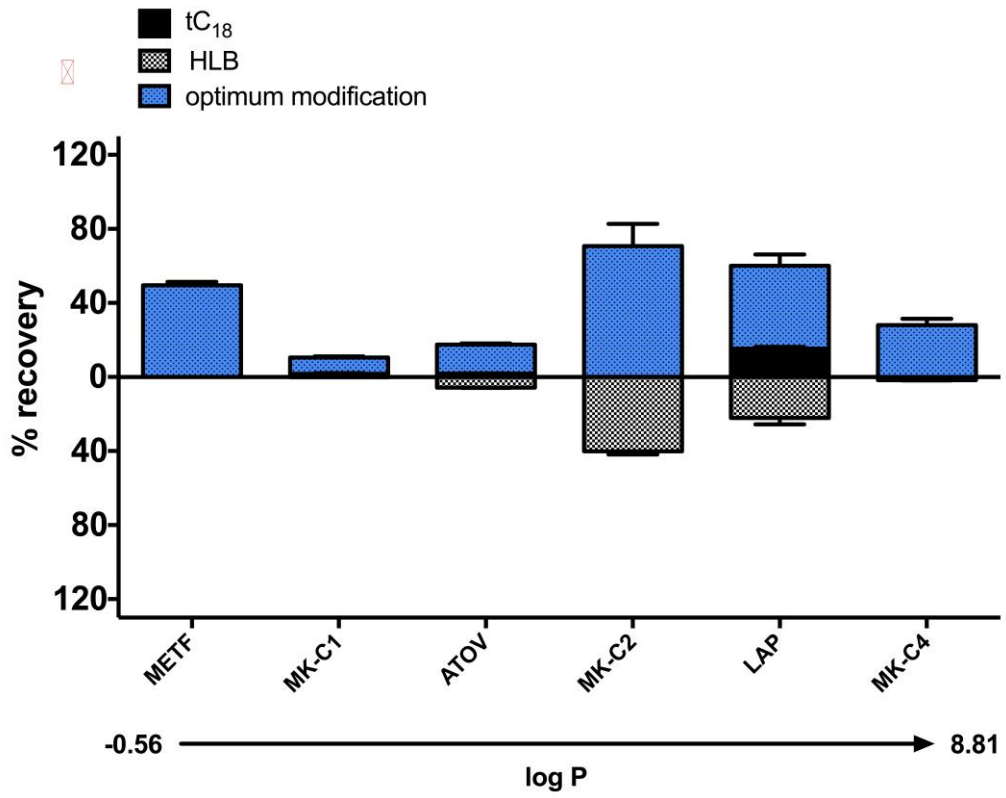

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1082 Figure 8

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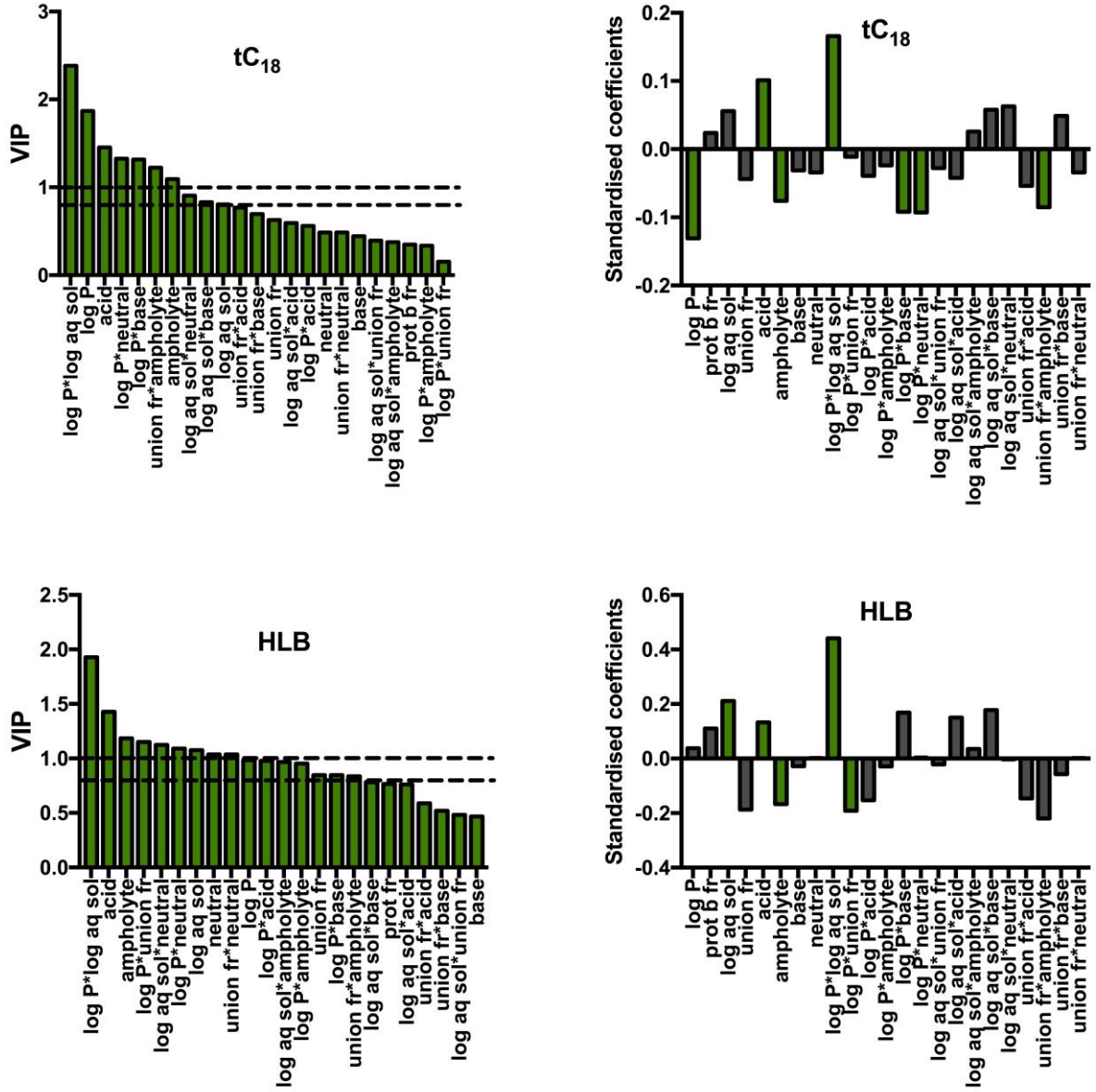

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1087 Figure 9

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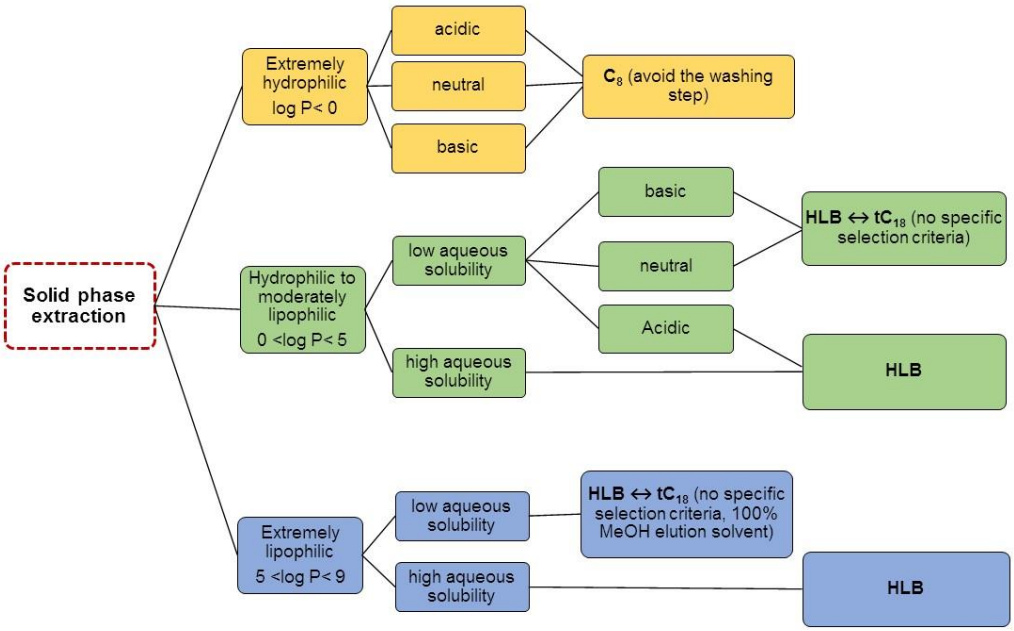

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1091 Figure 10